Phenotypic fingerprints of bacterium *Erwinia persicina* from larval gut of the diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae)

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Abstract: 【Aim】 Gut bacterium *Erwinia persicina* is one of the dominant bacterial species in the larval gut of the diamondback moth, *Plutella xylostella*. The aim of the present study is to investigate the phenotypic characteristics of this bacterium. 【Methods】 The cell phenotype of *E. persicina* was analyzed with BIOLOG Phenotype MicroArray (PM). Totally 950 different metabolic phenotypes were tested using the PM plates 1 – 10. 【Results】 *E. persicina* was able to metabolize 39.47% of the tested carbon sources, 89.74% of nitrogen sources, 100% of sulfur sources, and 100% of phosphorus sources. Most informative utilization patterns for carbon sources of *E. persicina* were organic acids and carbohydrates, and for nitrogen were various amino acids. The bacterium had 261 different nitrogen metabolic pathways and 95 different biosynthetic pathways. It had a wide range of adaptabilities, and could still metabolize in osmolytes with up to 9% sodium chloride, 4% potassium chloride, 5% sodium sulfate, 20% ethylene glycol, 6% sodium formate, 2% urea, 6% sodium lactate, 200 mmol/L sodium phosphate (pH 7.0), 20 mmol/L sodium benzoate (pH 5.2), 100 mmol/L ammonium sulfate (pH 8.0), 100 mmol/L sodium nitrate, and 100 mmol/L sodium nitrite, respectively. It also exhibited active metabolism under pH values between 4.5 and 10, with optimal pH around 7.0. The gut bacterium showed both decarboxylase and deaminase activities in the presence of various amino acids. 【Conclusion】 The phenotypic characterization of *E. persicina* increased our knowledge of the bacterium, in particular its interactions with insect hosts and the adaptability in gut environments, and showed us some possible approaches to controlling diamondback moth through decreasing *E. persicina* density.

Key words: *Erwinia persicina*; *Plutella xylostella*; phenotype microarray; metabolic fingerprint; gut bacteria

1 INTRODUCTION

The diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is one of the most destructive pests of cruciferous crops. It attacks economically important Cruciferae crops, including cabbage, cauliflower, broccoli, collards, mustard, rapeseed, radish and turnip (Talekar and Shelton, 1993). The annual cost associated with DBM damage and management worldwide was estimated to be 4 – 5 billion USD conservatively (Zalucki *et al.*, 2012). In Southeast Asia breakouts of this pest may cause a >90% loss to damaged crops (Indiragandhi *et al.*, 2007), and it has been a serious threat to vegetable production in China. Numerous studies have been conducted on DBM management strategies (Reddy and Guerrero, 2000; Shelton and Nault, 2004), mechanisms and control of pesticide resistance (Ji *et al.*, 2005; Gong *et al.*, 2013; Kim *et al.*, 2013), developmental biology (Kim and Kim, 2010; Martins *et al.*, 2012; Wu *et al.*, 2015), and herbivory and detoxification features (You *et al.*, 2013).

Insects are often infected by microbial symbiosis, mainly with bacteria. The bacterial associations generally play significant roles in morphogenesis, food digestion, nutrition, antifungal toxin production, pheromone production, regulation of pH, synthesis of vitamins, temperature tolerance, resistance against parasitoids, and detoxification of noxious compounds in the host insects (Dillon and Dillon, 2003; Genta *et al.*, 2006; Xia *et al.*, 2013b). A high diversity of bacteria has been reported from a number of herbivory insects, such as

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Tetanops myopaeformis, *Lymantria dispar*, *Melanoplus sanguinipes*, *Manestra brassica* and *Helicoverpa armigera* (Iverson *et al.*, 1984; Spiteller *et al.*, 2000; Broderick *et al.*, 2004; Xiang *et al.*, 2006). So far there have been a few reports on the symbiotic bacteria in DBM (Indiragandhi *et al.*, 2007; Xia *et al.*, 2013a). *Pseudomonas* sp., *Stenotrophomonas* sp., *Acinetobacter* sp., and *Serratia marcescens* were found to be common species in larval gut of prothiofos-resistant DBM population; however, *Brachybacterium* sp., *Acinetobacter* sp. and *S. marcescens* were generally found in its susceptible larvae, and *Serratia* sp. was found usually from its field-caught population (Indiragandhi *et al.*, 2007). Xia *et al.* (2013b) also reported that *Serratia* sp., *Enterobacter* sp., *Stenotrophomonas* sp. and *Myroides* sp. were the major gut bacteria of DBM larvae (Xia *et al.*, 2013a). In our previous study, we found one dominant gut bacterium, *Erwinia persicina*, from the larval gut of diamondback moth (Li *et al.*, 2015). However, to our best knowledge, this bacterium has not been characterized and its functions are not clear. Recently, techniques such as phenotypic analysis were used to investigate the metabolic phenotypic diversity of various microorganisms, which we thought could also be used to clarify the metabolic characteristics of *E. persicina* found in DBM.

At the turn of this century, a high throughput phenotype microarray (PM)/OmniLog system was developed by scientists of the company Biolog (Hayward, CA, USA) and nearly 700 metabolic phenotypes of the bacterium *Escherichia coli*, as a model cellular system, were assayed (Bochner *et al.*, 2001; Bochner, 2003). In this system, the microorganism cells are inoculated in the test medium containing tetrazolium dye and with different substances in 96-well plates, to analyze the use of carbon, nitrogen, sulfur and phosphorus substrates, and the biosynthetic pathways (Wang *et al.*, 2015a). Cell respiration and growth result in the reduction of the tetrazolium dye and lead to blue color. The intensity of the color, which is proportional to microbial growth, is recorded every 15 min by a CCD camera and analyzed by OmniLog software, which yields a quantitative analysis of all data. The software sketches kinetic data of color formation against time for each well and ascribes artificial color to each plot.

The present study aims to elucidate the phenotype of *E. persicina*, a symbiotic gut bacterium isolated from DBM larvae. The results would provide detail information of *E. persicina*, and valuable knowledge about insect-microbe interaction and survivability of the bacterium in the gut of *E. persicina*.

2 MATERIALS AND METHODS

2.1 Bacterial strain and culture conditions

Isolates of *E. persicina* obtained from our previous study were selected as the modal species in this study (Li *et al.*, 2015). The isolates were maintained on nutrient agar [NA: beef extract (0.3%), peptone (0.5%), and agar (1.5%) in water (w/v)] plates (de Vries *et al.*, 2001) at 28℃ in darkness in a controlled climate cabinet, and were stored as suspensions in 30% glycerol at −20℃ for long time storage. They were retrieved at 28℃ using the YPGA medium (peptone, 5 g/L; yeast extract, 5 g/L; glucose, 10 g/L; and agar, 16 g/L; pH 7.2).

2.2 Molecular identification of *E. persicina*

A loopful of the colonies of *E. persicina* isolates on NA agar plate was swabbed from the agar, re-suspended in 150 μL of distilled water, boiled for 12 min in an Eppendorf tube, cooled to room temperature on ice for 8 min, and centrifuged at 9 000 g for 2 min. The supernatant was utilized for PCR. The 16S rRNA gene of each colony was amplified by PCR using the forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR amplifications were conducted by a thermocycler (Biorad MyCycler™, BioRad, CA, USA) in a 30-μL reaction system containing 6 μL of boiled supernatant, 3 mmol/L $MgCl_2$, 0.5 μmol/L of each primer, 1 unit of *Taq* DNA polymerase (TaKaRa, Dalian, China), 200 μmol/L (each) deoxynucleoside triphosphate (dNTP) in 1 × PCR buffer. The reactions were performed as follows: 95℃ for 10 min, 35 cycles of 30 s at 95℃, 120 s at 63℃, and 1 min at 72℃, and a final extension at 72℃ for 10 min. Products (4 μL) were loaded on 1.0% agarose (Biowest®, Spain) gels, electrophoresed at 100 V for 30 min and checked under UV transillumination (254 nm). The 16S rRNA nucleotide sequences were sequenced by Shanghai Sangon Biotech Company and subjected to homology searches in DNA databases.

2.3 Phenotypic characterization

One isolate of *E. persicina* was chosen randomly from the strains identified above for this study. All materials, reagents and media for the phenotypic study were bought from Biolog. The isolate was streaked on Biolog Universal Growth medium plus Blood agar (BUG + B) plates and incubated at 30℃ in darkness for 48 h. Cells were scraped from the

surface of the plates and re-suspended in appropriate medium containing Dye Mix C; 120 μL of a 1∶200 dilution of an 85% transmittance cell suspension were added to each well of the PM plates. Plates 1 – 8, testing for phenotypes of carbon, nitrogen phosphorus, sulfur, as well as for biosynthetic pathways, and plates 9 – 10, testing for osmotic/ion and pH effects, were used in this study. IF-0 GN Base was prepared for PM plates 1 and 2. IF-0 GN Base plus 20 mmol/L sodium succinate, pH 7.1, and 2 μmol/L ferric citrate was utilized for plates 3 – 10. After inoculation under clean bench, plates were incubated in the OmniLog incubator for 72 hours. Data were collected every 15 minutes by the Biolog incubator, and analyzed using Kinetic and Parametric software (Biolog). Phenotype diversities were analyzed based on the area difference under the kinetic curve of dye formation. This experiment was conducted twice.

3 RESULTS

3.1 Molecular identification of *E. persicina*

The 16S rDNA analysis revealed that the recovered isolates ZZCG2-2 and ZZCG2-6 from our previous study belonged to one species, *E. persicina*, and presented a 99% similarity with the 16S rDNA gene sequences of *E. persicina*. The nucleotide sequences were submitted to the NCBI database and their accession numbers were available in the GenBank as KM434150 and KM434155.

3.2 Phenotypic characterization

Isolate of *E. persicina* tested in our study presented typical phenotypic fingerprint. The bacterium was able to metabolize 39.47% of the tested carbon sources (60/95 in plate PM 1 and 15/95 in plate PM 2), 89.74% of tested nitrogen sources (80/95 in plate PM 3, 95/95 in plate PM 6, 86/95 in plate PM 7, and 80/95 in plate PM 8), 100% of tested phosphorus sources (59/59 in plate PM 4, wells A2 – E12), and 100% of the tested sulfur sources (35/35 in plate PM 4, wells F2 – H12) (Fig. 1).

As indicated by the data from PM 1 and PM 2 (carbon sources), *E. persicina* was able to use 75 different carbon sources (Fig. 1), of which 68 compounds were effectively utilized, including L-arabinose, N-acetyl-D-glucosamine, D-galactose, L-proline, D-mannose, D-sorbitol, glycerol, D-mannitol, D-ribose, D-fructose, α-D-glucose, *etc.* (Fig. 1, Table 1). In comparison, around 115 compounds could not be utilized by the gut bacterium (Fig. 1).

Using the PM 3 plate, *E. persicina* was tested for its ability to grow on 95 different nitrogen sources (amino acids) (Fig. 1). Around 66 compounds were effectively utilized by this bacterium, including ammonia, nitrite, nitrate, urea, L-alanine, L-cysteine, D-valine, *etc.* (Table 2), while other compounds could not be used or poorly used (Fig. 1). Using the PM 5, *E. persicina* presented 95 different biosynthetic pathways (Fig. 1). Meanwhile using the PM 6, PM 7 and PM 8 plates, *E. persicina* was tested for its ability to grow on 285 different nitrogen metabolic pathways, and it was found to be able to metabolize 91.58% of the tested compounds (Fig. 1).

Plates PM 9 and PM 10 were used to test the bacterial growth under various stress conditions. *E. persicina* showed active metabolism with up to 9% sodium chloride, 4% potassium chloride, 5% sodium sulfate, 20% ethylene glycol, 6% sodium formate, 2% urea, 6% sodium lactate, 200 mmol/L sodium phosphate (pH 7.0), 20 mmol/L sodium benzoate (pH 5.2), 100 mmol/L ammonium sulfate (pH 8.0), 100 mmol/L sodium nitrate, and 100 mmol/L sodium nitrite (Fig. 1). When combined with various osmolytes at the treatment of 6% sodium chloride, *E. persicina* presented effective growth in all tests (plate PM 9, wells B1 to B12, and C1 to C12). The pH range where *E. persicina* exhibited active growth was between 4.5 and 10, with an optimal pH around 7.0. When combined with various amino acids at pH 4.5, *E. persicina* showed effective growth in all tests (plate PM 10, wells B1 to B12, C1 to C12, and D1 to D12) except for the amino acids of L-isoleucine (plate PM 10, well B10), L-leucine (plate PM 10, well B11), L-tryptophan (plate PM 10, well C6), L-methionine (plate PM 10, well D1), L-norleucine (plate PM 10, well D2), *p*-aminobenzoate (plate PM 10, well D5), 5-hydroxy tryptophan (plate PM 10, well D9) and trimethyl amine-N-oxide (plate PM 10, well D11). In comparison, when combined with various amino acids at pH 9.5, the gut bacterium presented active growth in all tests (plate PM 10, wells E1 to E12, F1 to F12, and G1 to G12) except for L-tryptophan (plate PM 10, well F6), L-norleucine (plate PM 10, well G2), agmatine (plate PM 10, well G4), cadaverine (plate PM 10, well G5), and tyramine (plate PM 10, well G9). In PM 10, wells B1-D12 and E1-G12 tested the decarboxylase and deaminase activities of the gut bacterium in the presence of amino acids at pH 4.5 and pH 9.5, respectively. *E. persicina* were found to have both activities (Fig. 1, plate PM 10). Meanwhile, this bacterium also presented active growth under pressures

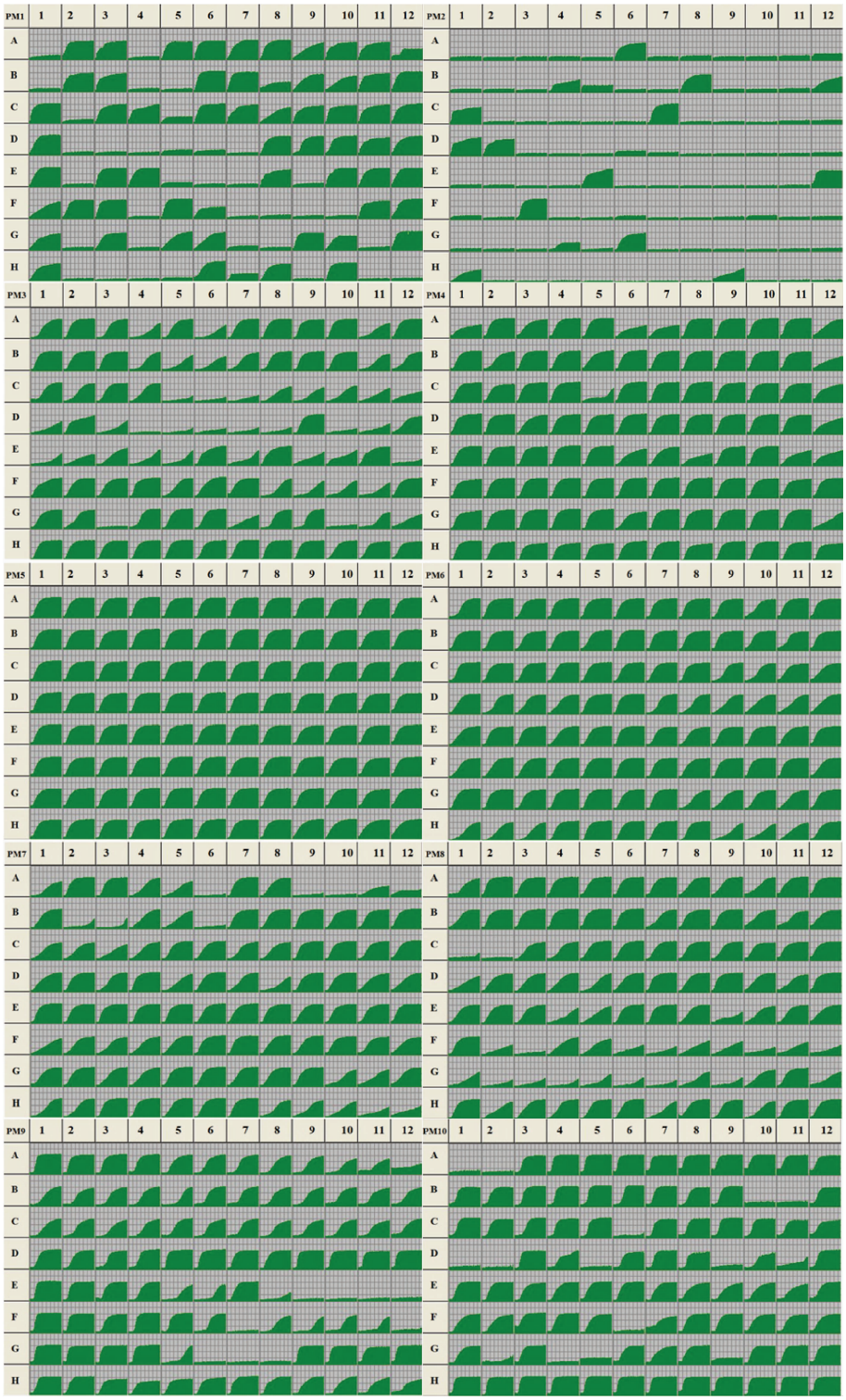

Fig. 1 Data for Biolog Phenotype MicroArray PM 1 – 10 plates of the gut bacterium *Erwinia persicina*

The numbers 1 – 12 on horizontal axis and the letters A – H on vertical axis are the layout of the tested Biolog MicroPlate. Utilization of the isolate of *E. persicina* from the gut of *Plutella xylostella* was indicated by green areas in the growth curve for each substrate.

of other compounds in the plate PM 10 (wells H1 to H12).

4 DISCUSSION

While a large number of studies have been conducted with *E. persicina* (Kiessling *et al.*, 2005; González *et al.*, 2007), its phenotypic diversity in insect gut is still poorly explored. Direct high-throughput assessment of phenotypes using the PM system (Bochner *et al.*, 2001) has received much attention in molecular biological, genomic, and population studies of microorganisms (Bochner, 2003;

Table 1 Substrates in PM 1 and 2 MicroPlates effectively metabolized by *Erwinia persicina*

Assay no.	Substrate	Assay no.	Substrate	Assay no.	Substrate
1-A02	L-arabinose	1-C09	α-D-glucose	1-G01	Gly-Glu
1-A03	N-acetyl-D-glucosamine	1-C10	Maltose	1-G03	L-serine
1-A05	Succinic acid	1-C11	D-melibiose	1-G05	L-alanine
1-A06	D-galactose	1-C12	Thymidine	1-G06	Ala-Gly
1-A07	L-aspartic acid	1-D01	L-asparagine	1-G09	Mono-methylsuccinate
1-A08	L-proline	1-D08	α-methyl-D-galactoside	1-G10	Methylpyruvate
1-A09	D-alanine	1-D09	α-D-lactose	1-G12	L-malic acid
1-A10	D-trehalose	1-D10	Lactulose	1-H01	Gly-Pro
1-A11	D-mannose	1-D11	Sucrose	1-H06	L-lyxose
1-B02	D-sorbitol	1-D12	Uridine	1-H08	Pyruvic acid
1-B03	Glycerol	1-E01	L-glutamine	1-H10	D-galacturonic acid
1-B06	D-gluconic acid	1-E03	D-glucose-1-phosphate	2-A06	Dextrin
1-B07	D,L-α-glycerol-phosphate	1-E04	D-fructose-6-phosphate	2-B08	Arbutin
1-B09	D,L-lactic acid	1-E08	β-methyl-D-glucoside	2-B12	3-O-β-D-galacto-pyranosyl-D-arabinose
1-B10	Formic acid	1-E10	Maltotriose	2-C01	Gentiobiose
1-B11	D-mannitol	1-E11	2′-deoxyadenosine	2-C07	β-methyl-D-galactoside
1-B12	L-glutamic acid	1-E12	Adenosine	2-D01	D-raffinose
1-C01	D-glucose-6-phosphate	1-F01	Gly-asp	2-D02	Salicin
1-C03	D,L-malic acid	1-F02	Citric acid	2-E05	D-glucosamine
1-C04	D-ribose	1-F03	M-inositol	2-E12	5-keto-D-gluconic acid
1-C06	L-rhamnose	1-F05	Fumaric acid	2-F03	Melibionic acid
1-C07	D-fructose	1-F11	D-cellobiose	2-G06	L-histidine
1-C08	Acetic acid	1-F12	Inosine		

Assay numbers represent the assays conducted on the Biolog PM 1-10 plates as in Fig. 1. The same for Table 2.

Table 2 Substrates in PM 3 MicroPlates effectively metabolized by *Erwinia persicina*

Assay no.	Substrate	Assay no.	Substrate	Assay no.	Substrate
3-A02	Ammonia	3-C01	L-tyrosine	3-F08	Thymine
3-A03	Nitrite	3-C02	L-valine	3-F12	Inosine
3-A04	Nitrate	3-C03	D-alanine	3-G01	Xanthine
3-A05	Urea	3-C04	D-asparagine	3-G02	Xanthosine
3-A07	L-alanine	3-C08	D-serine	3-G04	Alloxan
3-A08	L-arginine	3-C09	D-valine	3-G05	Allantoin
3-A09	L-asparagine	3-C10	L-citrulline	3-G06	Parabanic acid
3-A10	L-aspartic acid	3-C11	L-homoserine	3-G08	γ-amino-N-butyric acid
3-A11	L-cysteine	3-D02	N-phthaloyl-L-glutamic acid	3-G09	ε-amino-N-caproic acid
3-A12	L-glutamic acid	3-D09	Ethanolamine	3-G11	δ-amino-N-valeric acid
3-B01	L-glutamine	3-D12	Agmatine	3-H01	Ala-Asp
3-B02	Glycine	3-E03	Tyramine	3-H02	Ala-Gln
3-B03	L-histidine	3-E06	Glucuronamide	3-H03	Ala-Glu
3-B04	L-isoleucine	3-E08	D-glucosamine	3-H04	Ala-Gly
3-B05	L-leucine	3-E11	N-acetyl-D-glucosamine	3-H05	Ala-His
3-B06	L-lysine	3-F01	N-acetyl-D-mannosamine	3-H06	Ala-Leu
3-B07	L-methionine	3-F02	Adenine	3-H07	Ala-Thr
3-B08	L-phenylalanine	3-F03	Adenosine	3-H08	Gly-Asn
3-B09	L-proline	3-F04	Cytidine	3-H09	Gly-Gln
3-B10	L-serine	3-F05	Cytosine	3-H10	Gly-Glu
3-B11	L-threonine	3-F06	Guanine	3-H11	Gly-Met
3-B12	L-tryptophan	3-F07	Guanosine	3-H12	Met-Ala

Guard-Bouldin *et al*., 2007; Mols *et al*., 2007; Viti *et al*., 2007). Here, metabolic ability of an *E. persicina* isolated from *P. xylostella* was systematically analyzed using PMs and significant metabolic diversity was found.

Our results showed that a wide range of carbon compounds could be utilized and most of the nitrogen, sulfur, and phosphorus sources were also metabolized by *E. persicina*. These data indicated that this bacterium might have a great adaptability in the gut environment of *P. xylostella*. The most informative plates were PM 1/PM 2 (carbon sources), PM 3 (nitrogen sources), PM 9 (osmolytes conditions) and PM 10 (pH conditions). Corresponding findings have also been reported in other microorganisms (Friedl *et al*., 2008; Wang *et al*., 2015a, 2015b). Most informative utilization patterns for carbon sources were organic acids and carbohydrates, and for nitrogen sources were various amino acids. The strong metabolic abilities of *E. persicina* might play an important role in supporting the food digestion and body development of *P. xylostella*.

Additionally, the gut bacterium was able to adapt to a wide range of osmolytes and pH conditions. Decarboxylase of the bacterium generates alkaline amines by the catabolism of amino acids, which help to counteract an acidic pH. On the other hand, a high pH can be counteracted by deaminases that generate acids (Durso *et al*., 2004; Maurer *et al*., 2005). In this study, *E. persicina* showed high activities of deaminase and decarboxylase. This suggests that this bacterium can adapt to various gut pH and osmolyte conditions occurring during the host's feeding and maturity.

This study provided us some clues as to the development of novel approaches for the control of *P. xylostella*. Enhancing the amount of carbon and nitrogen sources that could not be metabolized, or decreasing the amount of sources that could be utilized by *E. persicina* in the food of *P. xylostella* might influence the density of this bacterium and subsequently depress the moth population. Important discoveries from analyzing phenotypes have been found in many microorganisms and their results have been successfully used in a wide range (Bochner and Giovannetti, 2008; Friedl *et al*., 2008; Gusarov *et al*., 2009). Meanwhile, changing the osmolytes or pH values in the gut to make it unsuitable for *E. persicina* may be also an approach to decrease its infection density. More research needs to be conducted to verify this hypothesis.

This study enriched our knowledge on the biology of *E. persicina*, in particular its interactions with insect hosts and the adaptability in gut environments, and showed us some possible approaches to controlling *P. xylostella* by decreasing *E. persicina* density.

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小菜蛾幼虫肠道细菌桃色欧文氏菌的代谢表型组学分析

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摘要：【目的】肠道细菌桃色欧文氏菌 *Erwinia persicina* 是小菜蛾 *Plutella xylostella* 幼虫肠道的优势细菌，本研究旨在阐明桃色欧文氏菌的代谢表型特征。【方法】采用 BIOLOG 细胞表型芯片技术系统研究了桃色欧文氏菌的细胞表型；采用 PM 1 – 10 代谢板，对桃色欧文氏菌的 950 种代谢表型进行了测定。【结果】桃色欧文氏菌能代谢 39.47% 的碳源、89.74% 的氮源、100% 的硫源和 100% 的磷源；高效代谢的碳源为有机酸类和碳水化合物类，高效代谢的氮源为氨基酸类。该肠道细菌表现出 261 种不同的氮源代谢通路和 95 种生物合成通路。桃色欧文氏菌具有广泛的适应性，能在分别具有高达 9% 氯化钠、4% 氯化钾、5% 硫酸钠、20% 乙二醇、6% 甲酸钠、2% 尿素、6% 乳酸钠、200 mmol/L 磷酸钠（pH 7.0）、20 mmol/L 苯甲酸钠（pH 5.2）、100 mmol/L 硫酸铵（pH 8.0）、100 mmol/L 硝酸钠和 100 mmol/L 亚硝酸钠的渗透溶液中正常代谢；其适应 pH 值范围为 4.5 ~ 10，最适 7.0。在多种氨基酸的作用下，桃色欧文氏菌均表现出脱羧酶和脱氨酶活性。【结论】桃色欧文氏菌的代谢特征增加了我们对该肠道细菌，特别是其与宿主昆虫的互作及其在肠道环境中的适应性的认识，同时表明通过降低桃色欧文氏菌密度来防治小菜蛾的可能性。

关键词： 桃色欧文氏菌；小菜蛾；表型芯片；代谢指纹图谱；肠道细菌
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